WGSMART Draft Guidelines for Image Quality in SmartDots Events

WGSMART

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The final guidelines will be published in the **ICES User Handbook**
series in December 2023

Contents

Introduction

SmartDots is the tool officially endorsed and recommended by WGBIOP for preparing and conducting exchanges and workshops on age reading, maturity staging, and fish egg and larvae identification. Through SmartDots coordinators can easily set up and manage dedicated events involving multiple readers across several institutes, following a streamlined protocol.

WGBIOP has already released guidelines for both age reading and maturity staging exchanges, and will be providing guidelines for egg and larvae identification in the future. However, those documents focus on the coordination of events and the proper methodology for participants, rather than specific details for providing sample images of appropriate readability. Furthermore, wishes to use the SmartDots tool for reference sets have been raised, and development of such functionalities are being outlined. Image quality is crucial to ensure that users can confidently annotate the images and that calibration results reflect the users expertise, are non-biased and interpretable. For these reasons images provided through SmartDots should be standardized as much as possible.

Providing unique technical recommendations for sample preparation and image acquisition is at present not possible due to the variety of equipment used in institutes laboratories. Instead, this document presents general guidelines to be taken into consideration while producing image samples for an event, and each institute should in turn develop its own internal routines to get similar results with their available equipment.

SmartDots file recommendations
The SmartDots software accommodates various file types. It is generally recommended to provide full resolution images in compressed formats such as JPG, with a file size of 5Mb or less.

Image size can have a significant effect on loading times. If larger images or if images of the highest quality are required to interpret the smaller details then users need to be made aware that longer loading times should be expected. Usually, simply converting an image to JPG at 80 to 90% quality is enough to reduce file size to 1-5Mb without any noticeable effect on image readability. Such conversions can be made for a collection of images by using common image editing software, like ACDSee on Windows OS, Photos on Mac OS, and Darktable on Linux, or an R-script.

Guidelines for imaging of age reading material

Age reading events in SmartDots are primarily done on images of otoliths and scales but can involve other calcified structures such as spines.

Image of whole otoliths under water of *Trachurus trachurus* (reflected light)

Image of whole otoliths of *Mullus surmuletus* under water (reflected light) with the right otolith burnt.

Image of otolith transverse section of *Solea solea* embedded in resin (transmitted light)

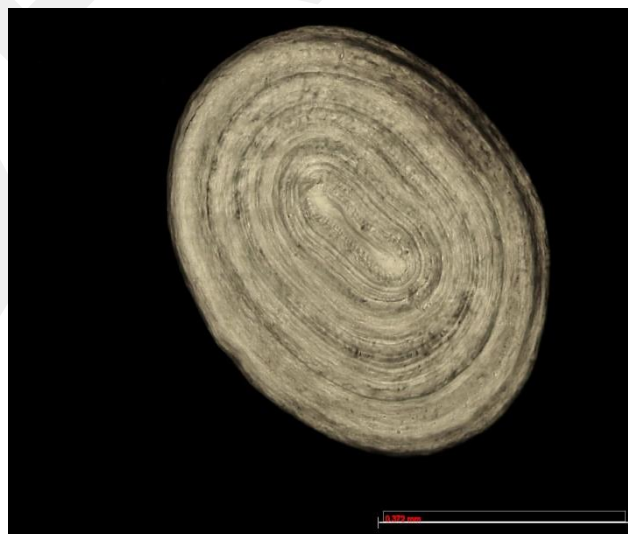Example of scale of *Sparus aurata* under water (transmitted light)

Image of filaments rising section of *Lophius piscatorius* embedded in resin (reflected light).
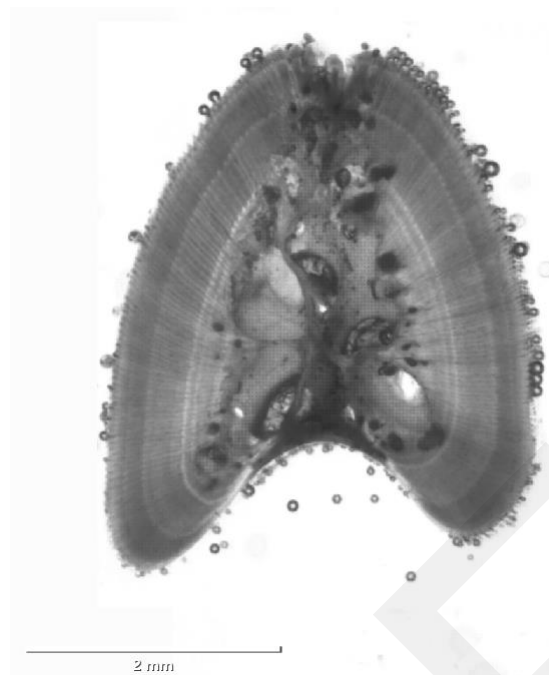
Image of spine section of *Thunnus albacares* embedded in resin (transmitted light)

These age structures can be prepared in several ways and each comes with its own specificities. Some examples include otolith transverse sections embedded in resin or epoxy, whole otoliths broken and placed vertically in modelling clay, imaged whole submerged under a liquid, broken and burned, or processed with a specific staining agent. They may be imaged under reflected (from above), transmitted (from below) or light from the side.

General quality guidelines
- Proper sample preparation is vital as it is important to ensure as clear a sample as possible. All left over tissue needs to be removed, dust specks and air bubbles avoided.

- Ensure that there are no strong light sources causing flare on your samples, such as sun shining through a window or an overhead light source.

- The most important features of a calcified structure for age reading are the annuli. Where possible all areas of the structure being read should be in focus. Sometimes this is not always possible with larger samples. In such cases, the main focus area should be the primary reading axis.

- Ensure the background is a uniform colour

- Correct image contrast ensures the best distinction between opaque and translucent zones.

- Exposure level is important. Overexposure (too much light) will result in whites bleeding onto the darker areas. Underexposure (too little light) will result in the inability to accurately distinguish opaque from translucent zones. The SmartDots software provides basic brightness and contrast tools but it essential that the images are of the required quality prior to being uploaded.

- As recommended within the SmartDots user manual, all images should feature a visible and correctly calibrated scale bar. Correct calibration ensures that readers can rely on the images

magnification and that any measurements taken of features within the otolith are correct. An image of a calibration stick taken with the same magnification as the otolith images and showing the scale bar that matches the scale bar on the otolith images should be saved with each set of images.
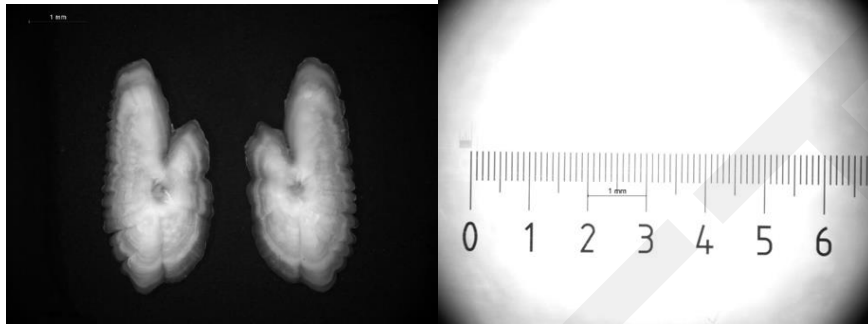
Otolith and calibration image examples

In order for the software to automatically detect the scale bar on the image:
- A horizontal scale bar needs to be present on the picture
- The scale bar must be an uninterrupted line (no dashed line)
- The colour of the scale needs to be in high contrast with the image (e.g. white scale line on a dark picture, black scale bar on a bright picture)
- The scale value must appear above the scale bar and not too far from it in a good readable font
- The scale value must contain a number and a unit. Examples are: 1 mm, 100 μm

- For a set of images for a given species keep the same image size or rather the same XY image field to not bias the interpretation with changes in magnification. The same for the camera settings (color, metric, resolution...)
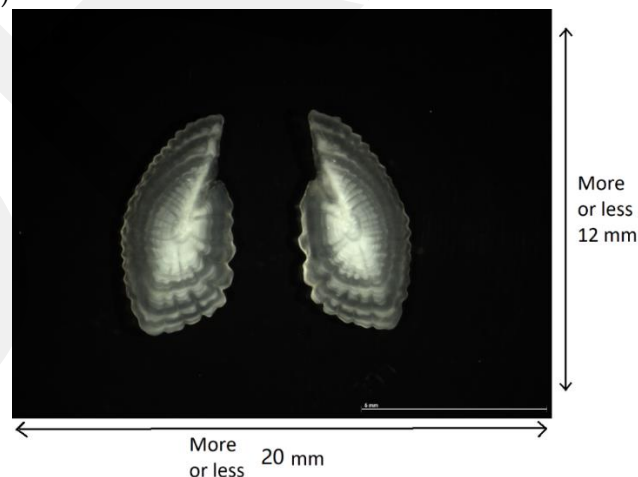

Image with a standardized image field for this *Trachurus trachurus* species in x ~20mm and y ~12mm.

- Coordinators should ensure, if possible that all images are taken within the same institute to ensure similar equipment, acquisition parameters and scale across the samples. If this is not possible then the previous point (providing a calibration image) needs to be followed by each institute providing images.

- Unless a specific preparation technique requires so, colour information is generally not needed and images in grayscale provide the clearest rings. They have less information, thus the files are smaller at the same resolution, which can help ensuring the images are within the recommended size for optimal loading time in SmartDots without significant downgrading of quality.

Image optimisation
Most image capturing software has features for improving exposure, contrast and gain.

Images can be processed to maximise readability (see for example Campana *et al.*, 2016). Conversion to grayscale, adjustment of midtones levels, slight increase in contrast and brightness, and a light sharpening filter are usually enough to get very clear images.
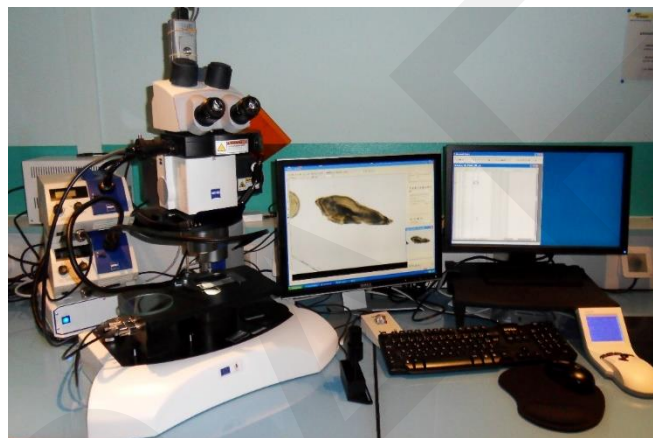

Example of image acquisition of otolith in transmitted light by binocular associated with a camera and an image acquisition and processing software.

General recommendations:
- Ensure an experienced age reader for the species in question is involved in image acquisition
- Coordinators should provide a few test images early on so a reader can assess whether those are suitable for the event (before investing the time and resources needed to image the whole batch of samples).
- Take a calibration image with each set of images

Issues to avoid when preparing and imaging samples
- Tissue remains on the otolith
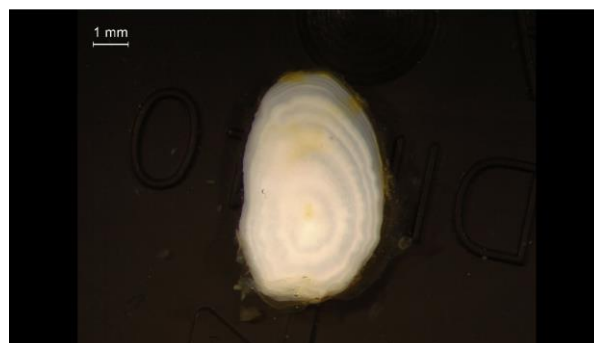
- Multiple bubbles or material on the sample surface which leads to the sample structure being obscured and/or out focus. Under reflected light, shadows are projected onto the structure which blend with translucent zones and may lead to errors in annotating. This may be difficult to detect before the image is reviewed in full screen.
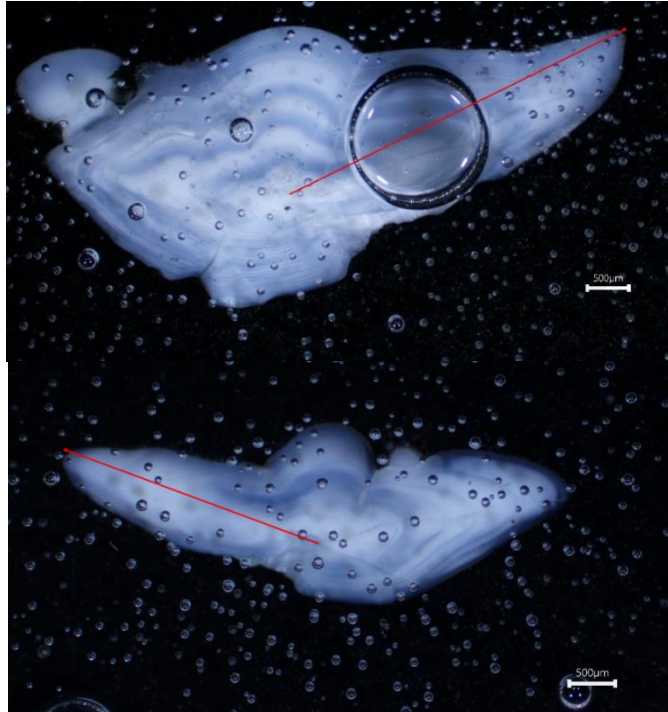
- Focus plan not on the sample itself. This comes often in tandem with the previous error, where the camera may pick up focus on the bubbles floating above the sample, resulting in out of focus blurry rings.

- Sample is difficult to see due to reflections on the immersion or mounting medium.

- Sample edge difficult to see due to or lack of contrast with the background. This can be particularly problematic when some samples in a batch are much smaller than others, resulting in images with comparatively a larger proportion of background. It may be necessary to increase the exposure when taking images of smaller samples.

Image naming

In order for the application and the associated analyses to work properly, it is necessary that the image names respect several criteria:

- That the beginning of the image name matches the sample ID because the sample ID is used to match the image to the sample.
- That the name of the image is unique in the series used for the event,

The following points are important if splitting the samples from the same event into different analyses and reports (e.g. by preparation method, type of structure, light type used…)

- That the code for the preparation method used is present in the name. See Link to this CodeType: //vocab.ices.dk/?ref=1511
- That the code for the calcified part used is present in the name
- That the light type used is present in the name. e.g "R" for reflected light and "T" for transmitted light

Here is an example of how to name your images if you want to add more than one image of an otolith:

PLE_2020_Q1_7A_52124_005_T (for transmitted light)
PLE_2020_Q1_7A_52124_005_R (for reflected light)

Guidelines for imaging of maturity staging material

Maturity staging is done on images of the whole individual and its gonads at the macroscopic scale, with histological slides supplemented whenever available. One challenge with maturity staging of pictures is that it is impossible to physically interact with the sample, which is often done as to assess firmness and grain of the gonads. This is particularly important to distinguish between stages B and C of the ICES SMSF scale (https://vocab.ices.dk/?CodeID=201781).

State	Stage	Possible sub-stages
SI. Sexually immature	A. Immature	
SM. Sexually mature	B. Developing	Ba. Developing but functionally immature (first-time developer)
		Bb. Developing and functionally mature
	C. Spawning	Ca. Actively spawning
		Cb. Spawning capable
	D. Regressing/ Regenerating	Da. Regressing
		Db. Regenerating
	E. Omitted spawning	
	F. Abnormal	

Official ICES SMSF (sexual maturity stage for fish), (ICES, 2018)

Multiple images should be taken per individual fish, at least one of the whole animal and another of the gonads (either external observation or after dissection) (Figure 1). To standardize the images place the specimen on the right side, so that the fish is facing left. All images should contain a clear tag with the unique fish number on it. If possible, sampling time, area, length and species can also be provide on the tag, but these data should be available in the fish information in SmartDots. For flatfish species at least 6 macroscopic images should be taken, while for round fish at least 4 macroscopic images are required (see below). For elasmobranch species, at least 3/4 macroscopic images should be provided (see below). Images of histological sections should be used for validation of the macroscopic maturity staging. For each individual fish at least 2 histological images are required.

Photographing gonads

Example 1: Fish with the gonad inside

Example 2: Fish with the gonad lying next to it

Example 3: close-up of the gonad

Figure 1. Example of multiple images per specimen. Source: Illustrated protocol for maturity staging provided to WKMSGAD 2014.

Macroscopic images for flatfish

1. The first image should feature the whole flatfish on the dorsal side on a measuring board with visible gonads, as scale and ratio of gonad to body length are primary features for staging.

2. Detail image of picture 1, flatfish on the dorsal side zoomed in on the gonads. Show the pressure characteristic on the picture to see if the fish is running. As it is difficult to show the pressure test for male specimens, a homogenous background is needed as long as it is not white.

3. This image should feature the whole flatfish on the ventral side on a measuring board with visible gonads.

4. Detail image of picture 3, flatfish on the ventral side zoomed in on the gonads. Show the pressure characteristic on the picture to see if the fish is running.

5. The gonads should be removed delicately and photographed outside the fish, placed on a measuring board, allowing to view the gonads in finer detail, e.g. blood vessels, transparency, number and aspect of individual oocytes in females.

Macroscopic images for round fish
1. The first image should feature the whole round fish on the ventral side on a measuring board with visible gonads.

2. Detail image of picture 1, round fish on the ventral side zoomed in on the gonads. Show the pressure characteristic on the picture to see if the fish is running. As it is difficult to show the pressure test for male specimens, a homogenous background is needed as long as it is not white.

3. The gonads should be removed delicately and photographed whole outside the fish, placed on a measuring board, allowing to view the gonads in finer detail, e.g. blood vessels, transparency, number and aspect of individual oocytes in females.

4. Picture of a longitudinally cut of the gonad.

Macroscopic images for Elasmobranchs
1. The first image should feature the whole Elasmobranch specimen on the ventral side on a measuring board with visible reproductive apparatus (ovaries (O), oviducal glands (OG) and uteri (U) in females and testes (T), deferens ducts (DD) and claspers (CL) in males) (Figure 2).
2. Detail image of picture 1, Elasmobranch specimen on the ventral side zoomed in on the reproductive apparatus.
3. The reproductive apparatus should be removed delicately and photographed whole outside the fish, placed on a measuring board allowing to view all details, e.g. blood vessels, number and aspect of individual oocytes, dimension of oviducal glands, in females and deferens ducts in males.
4. When females have egg-cases (EC) (in oviparous species) or candle-case (CC) and/or embryos (in viviparous ones) inside the abdominal cavity, un image should be reported.
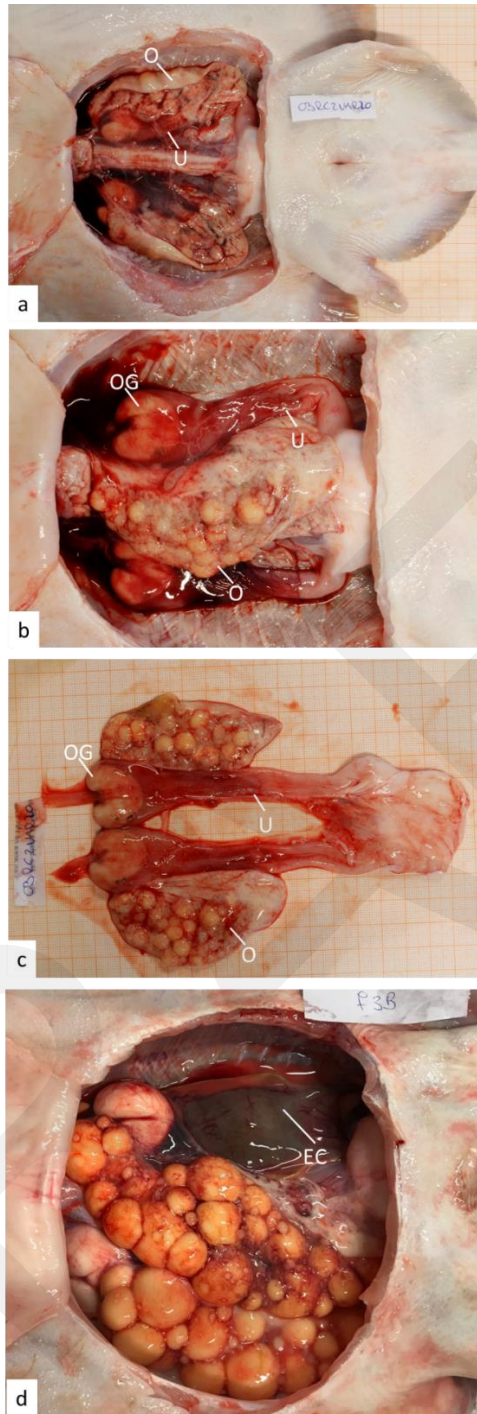
Figure 2. Example of an oviparous female of *Raja clavata* on the ventral side with the whole reproductive apparatus visible (a), ventral side zoomed in on the reproductive apparatus (b), reproductive apparatus outside the abdominal cavity (c). In Figure d a female with egg-cases inside the abdominal cavity is reported. EC, egg-case; O, ovary, OG; oviducal gland; U, uterus.
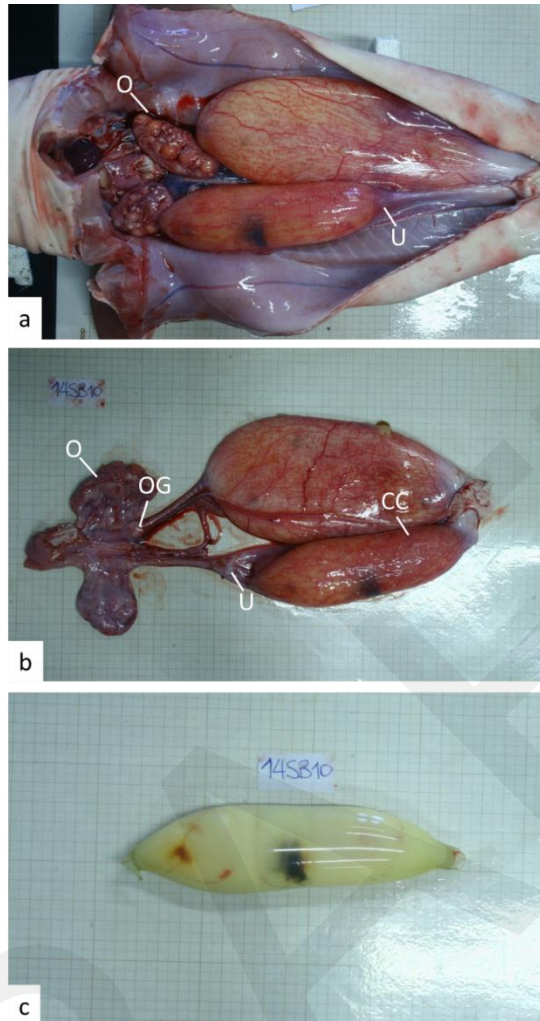
Figure 3. Example of a viviparous female of *Squalus blainville* on the ventral side with the whole reproductive apparatus visible (a), reproductive apparatus outside the abdominal cavity (c). In Figure d, a detail of a candle cases outside the abdominal cavity is reported. CC, candle-case; O, ovary, OG; oviducal gland; U, uterus.
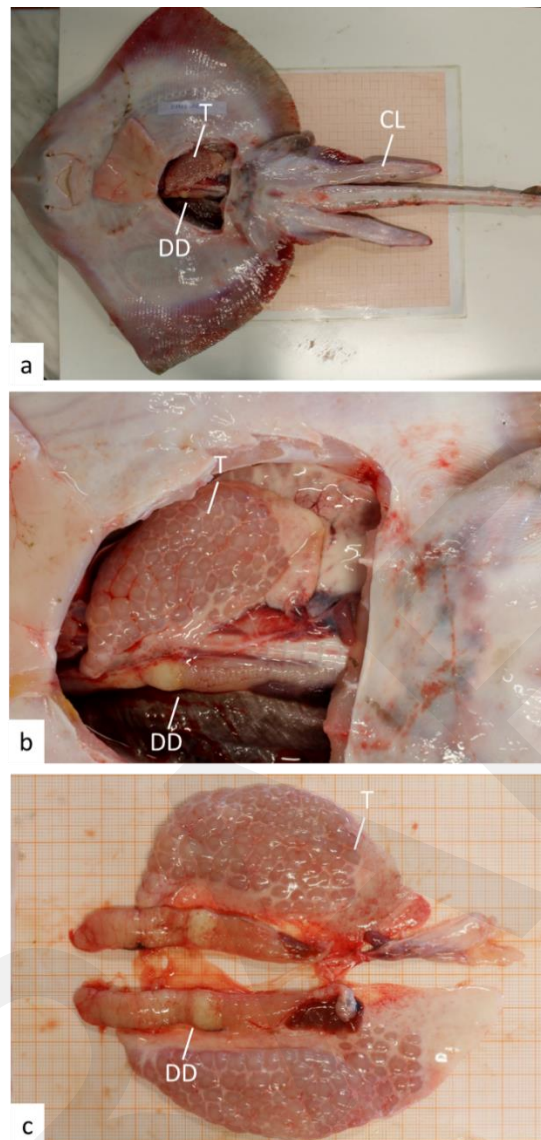
Figure 4. Example of male of *Raja clavata* on the ventral side with the whole reproductive apparatus and claspers visible (a), ventral side zoomed in on the reproductive apparatus (b), reproductive apparatus outside the abdominal cavity (c). CL, clasper; DD, deferens duct; T, testis.

Histological images for fish

1. The first image should give an overview of a whole cross section of one gonad. The gonad wall should be visible to determine the thickness of the wall. The image should contain a scale bar (Figure 5)

2. Detail image of picture 1, where oocytes or sperms cells in different development stages are visible. The image should contain a scale bar in order to be able to measure oocyte size. For males the sperm duct should be visible to be able to determine if sperm is running or not (Figure 5).
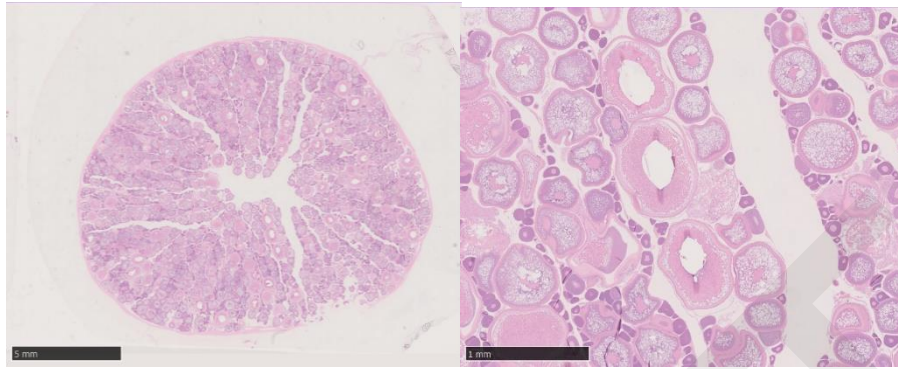
Figure 5. Whole cross section and detailed histological image of the ovary on the left and right respectively.


General quality guidelines

- For fish species where macroscopic maturity staging is done on fresh fish pictures should be taken on fresh fish whenever possible for the best quality. Older or frozen/thawed samples have a distinct appearance and can be significantly harder to distinguish. For species where frozen or thawed fish are used for macroscopic maturity staging pictures should be taken of frozen and or thawed specimens. Information on fresh, frozen or thawed status of the specimen in the image should be clearly stated in the available data, but preferably also on the tag in the image.
- Preferable image acquisition should be done on adequate and comfortable space like a laboratory, avoiding doing it in fishing vessels.
- Ensure the gonads are clean and clearly identifiable, especially when imaging them dissected: carefully clean away excess blood and tissues, but do not wash them as this will also wash away running oocytes or sperm cells, to leave only the gonads clearly demarcated.
- As recommended within the SmartDots user manual, all images should feature a visible and correctly calibrated scale bar.
- Use manual focus to ensure that the surface of the gonads is uniformly in focus.
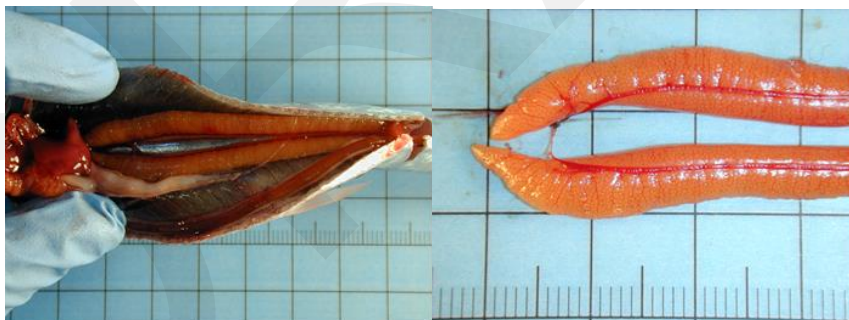
Example image of bony fish gonads photographed *in-situ* and after removal.
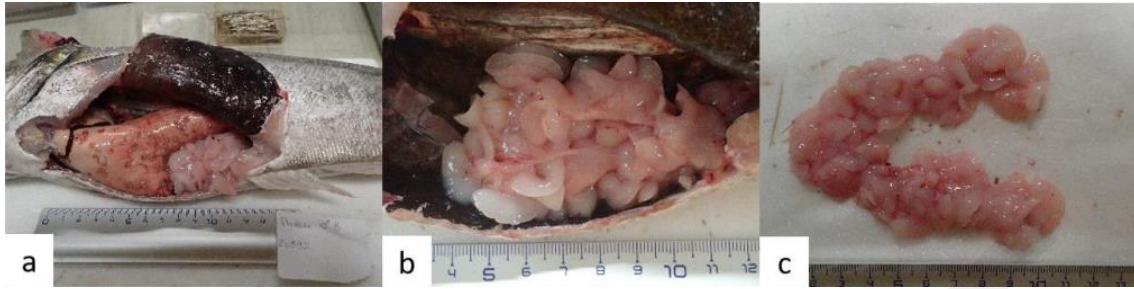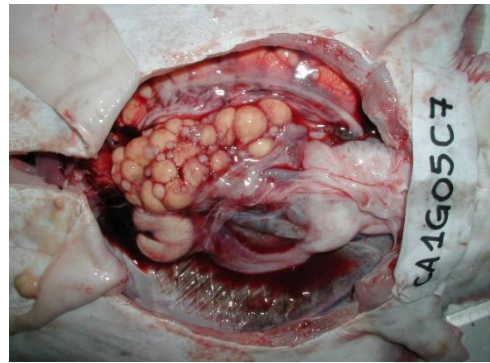
The 3 types of image : a) gonads inside the fish with other organs; b) gonads inside the fish without organs; c) gonads outside the fish (*Le Meleder Anna 2022*)
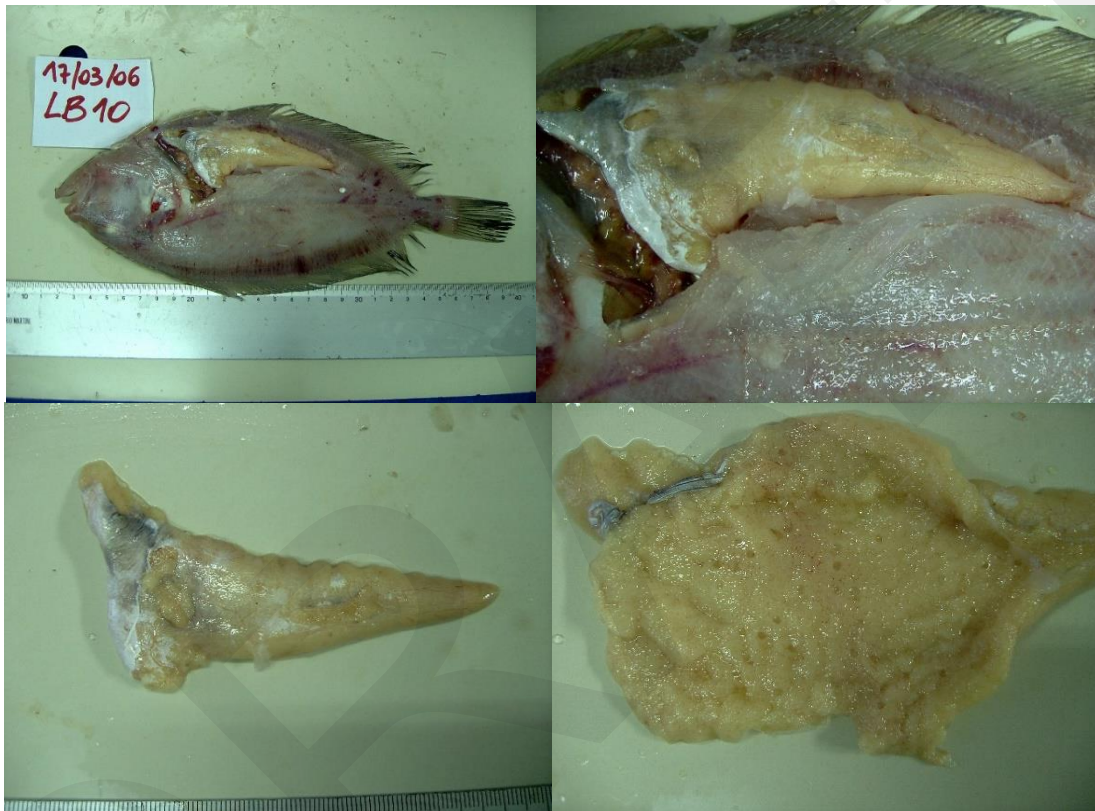
Images showing flatfish gonads in-situ and after removal

Different types of particular case images: a) with knife; b)individuals with milt; c) open gonads (*Le Meleder Anna 2022*)
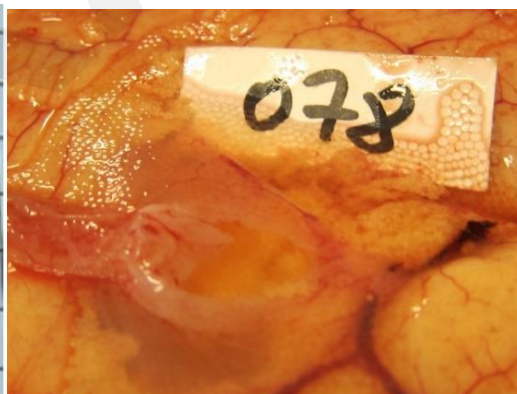
Example image of elasmobranch gonads photographed as whole individual and after dissection.

Issues to avoid when preparing and imaging maturity samples

Sample is not adequate because light reflection and dirt. Proportion of gonad/space in the image is not adequate. Source: Image provided to WKMSGAD 2014.

Sample is not properly focused and there is no size of the grid behind. Source: Image provided to WKMSGAD 2014.

Guidelines for imaging of larval identification

Total or standard length of the fish larva must be measurable on an image. The tail fin and notochord should be clearly visible. A scale bar should be present on the image. For identification, the present fins and pigmentation should be visible. Especially for Clupeidae (but this can also be helpful for other species), the myomeres and pylorus should also be visible.

General quality guidelines

1) If possible, select a specimen which is not extremely bend or damaged, make sure all important traits are visible (fins, fin rays, gut, notochord, etc.)
2) Photograph the specimen in a container filled with transparent fluid (water, ethanol, glycerin etc.) and place it in the middle (within the focus point) (see example image)
3) Place the specimen on its right side, so that it is facing left
4) If the specimen is bended and does not lie flat, you can weight it down gently using a coverslip
5) If the specimen still does not lie flat or you don´t want to damage it by using the coverslip, you can gently turn the container in which the specimen is lying by stacking objects underneath it at different angles (see example image)
6) If you want to fix the specimen further, so that it can be photographed at a certain angel, you can use knead to mold the desired shape in which the fish fits perfectly
7) Use a black and a white background so all traits are visible e.g., fin rays are often not visible when using a white background.
8) Further illumination using different lightning sources can be provided by installing more light sources directed at the specimen, with light coming from various angles.
9) If all planes of the object are supposed to be clear pictures with different focus points can be taken and later stacked (e.g., using Helicon Focus Software)
10) A good resolution is necessary to zoom in on the identification criteria (myomeres, pylorus, fins, pigments, globules).
11) As recommended within the SmartDots user manual, all images should feature a visible and correctly calibrated scale bar. Correct calibration ensures that experts can rely on the images magnification and that any measurements taken of features within the larva and eggs are correct. An image of a calibration stick taken with the same magnification as the larva and eggs images and showing the scale bar that matches the scale bar on the larva and eggs images should be saved with each set of images. **In order for the software to automatically detect the measure the scale bar on the image:**
    - A horizontal scale bar needs to be present on the picture
    - The scale bar must be an uninterrupted line (no dashed line)
    - The colour of the scale needs to be in high contrast with the image (e.g. white scale line on a dark picture, black scale bar on a bright picture)
    - The scale value must appear above the scale bar and not too far from it in a good readable font
    - The scale value must contain a number and a unit. Examples are: 1 mm, 100 μm

Examples of a specimen in a water filled container, use of moulds to tilt the container to get the specimen flat on the image, and photographing setup with lighting.

Three images of the same 9.2 mm TL *Sardina pilchardus* larva viewed at different light sources and illuminations. Top: darkfield illumination, middle: in polarized light for myomere counts, bottom: top light against white background for pigmentation. Scale bars had been cut out from the original images and are not shown.
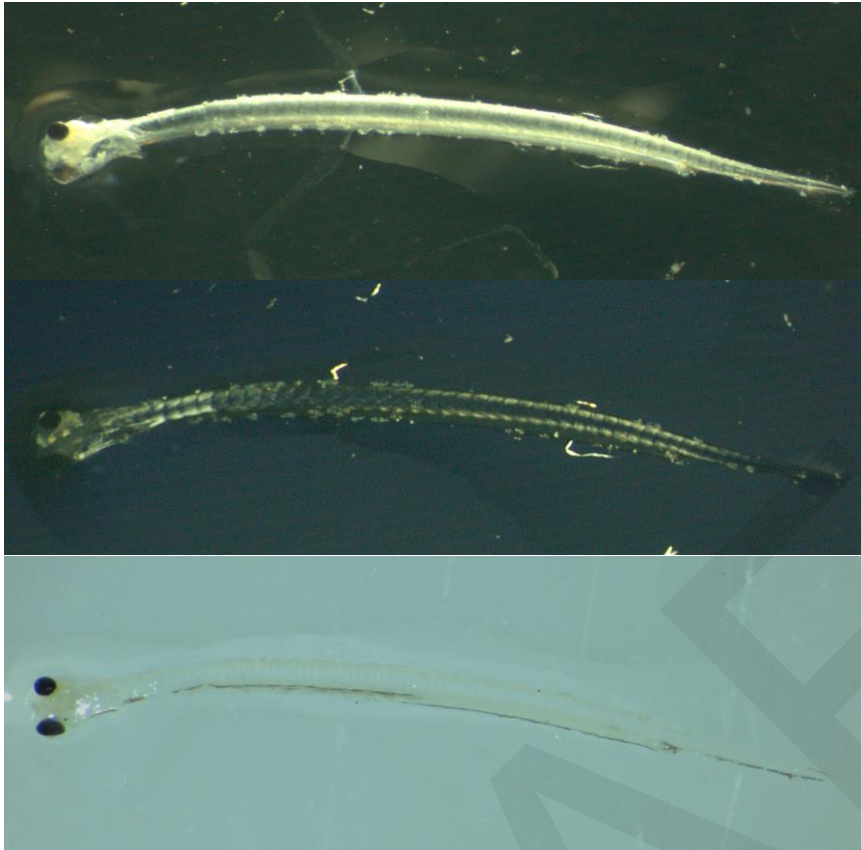
Three images of the same 8.6 mm TL *Sprattus sprattus* larva viewed at different light sources and illuminations. Top: darkfield illumination, middle: in polarized light for myomere counts, bottom: top light against white background for pigmentation. Scale bars had been cut out from the original images and are not shown.

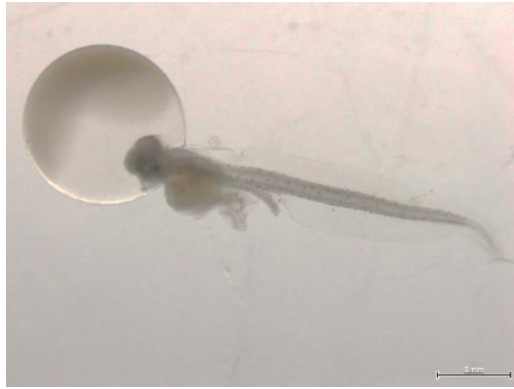Image of *Solea solea* (transmitted light)

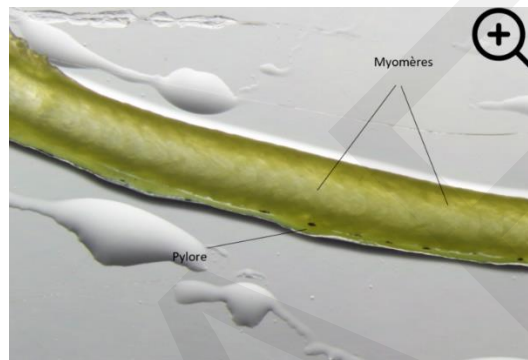Image of *Limanda limanda* (transmitted light)

Image of zoom in (transmitted light)

Image of zoom in (transmitted light)

Issues to avoid when preparing and imaging samples

Example of unclear and dark image (transmitted light)

Example of unclear and dark image (transmitted light)

Guidelines for imaging of eggs identification

A fish egg must be measurable on a picture. The membrane, the embryo if present, the pigments and oil globules must be clearly visible. The scale bar should be provided on the image.

General quality guidelines
- Proper sample arrangement is vital as it is important to get as clear a sample as possible without overlap of eggs.
- A good resolution is necessary to zoom in on the identification criteria (presence of oil globules, perivitelline space, sculptured membrane, pigments,).
- Make sure the bottom is as uniform as possible
- Take images with different light sources, transmitted light or top light to make sure all identification criteria are visible.
- A high contrast and optimal sharpness is required on the whole sample.
- As recommended within the SmartDots user manual, all images should feature a visible and correctly calibrated scale bar. Correct calibration ensures that experts can rely on the images magnification and that any measurements taken of features within the larva and eggs are correct. An image of a calibration stick taken with the same magnification as the larva and eggs images and showing the scale bar that matches the scale bar on the larva and eggs images should accompany each set of images.

In order for the software to automatically detect the measure the scale bar on the image:
- A horizontal scale bar needs to be present on the picture
- The scale bar must be an uninterrupted line (no dashed line)
- The colour of the scale needs to be in high contrast with the image (e.g. white scale line on a dark picture, black scale bar on a bright picture)
- The scale value must appear above the scale bar and not too far from it in a good readable font
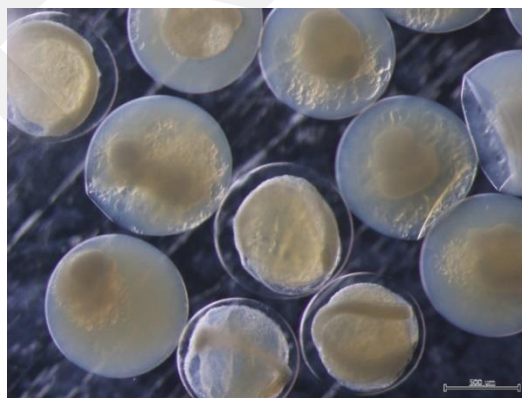- The scale value must contain a number and a unit. Examples are: 1 mm, 100 μm

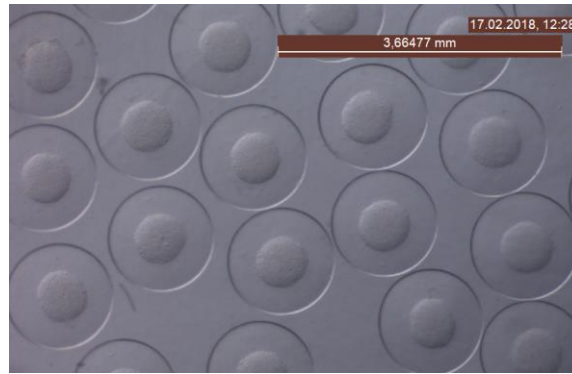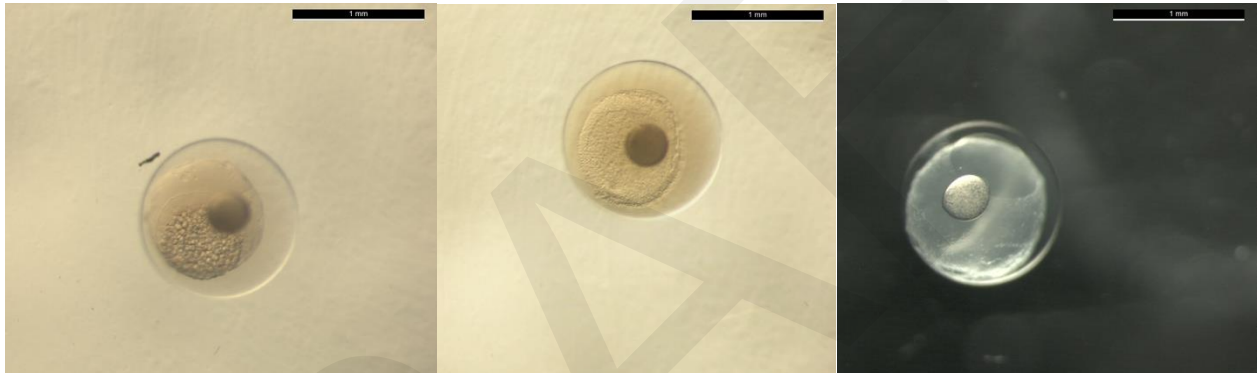Image of *Sprattus sprattus eggs* (darkfield illumination)

Image of cod (*Gadus morhua*) eggs (stage 1a in low transmitted light)

Three images of mackerel eggs, stage 1a, 1b and 2, left to right. The diagnostic features, which distinguish all three stages from each other, are clearly visible: Cleavage had produced a cell bundle where single cells are still visible in the stage 1a egg. In the stage 1b egg, the blastodisc, visible as a 'signet ring', had just formed – high contrast is needed here to visualize the double cell layer, which forms the 'signet ring'. In the stage 2 egg the blastopore is still wide open and an embryo in early development is visible – again, high contrast is needed to visualize both, the developing early embryo and the blastopore edges.
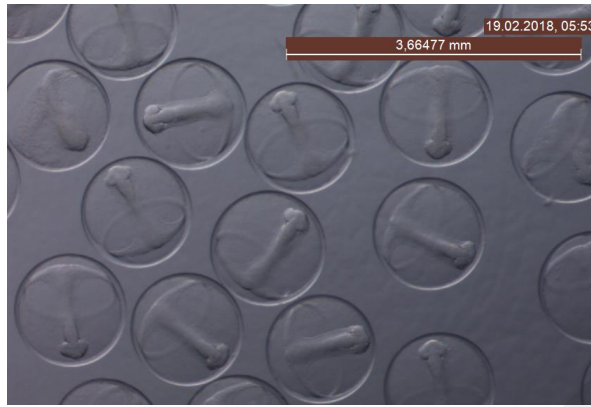
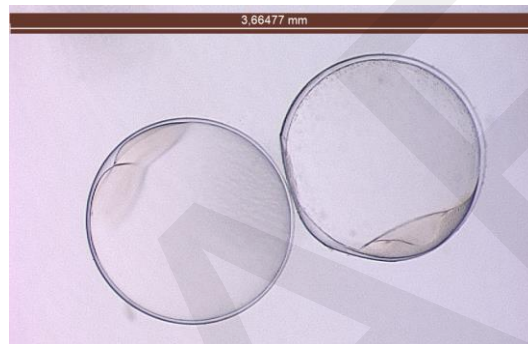Image of cod (*Gadus morhua*) eggs (stage 2 in low transmitted light)

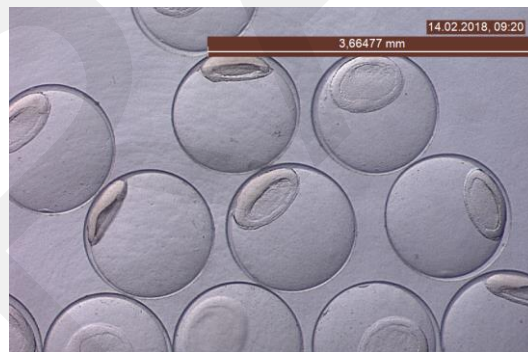Image of haddock (*Melanogrammus aeglefinus*) eggs (early stage 1a in transmitted light)

Image of haddock (*Melanogrammus aeglefinus*) eggs (early stage 1b in transmitted light)

Image of haddock (*Melanogrammus aeglefinus*) eggs (stage 2 in transmitted light)

Non-preferred images:

Image of an unknown fish egg (transmitted light too low)

References

Campana, S. E., Valentin, A. E., MacLellan, S. E., and Groot, J. B. 2016. Image-enhanced burnt otoliths, bomb radiocarbon and the growth dynamics of redfish (Sebastes mentella and S. fasciatus) off the eastern coast of Canada. Marine and Freshwater Research, 67: 925–936.

https://lm-anna.github.io/MaturityScaleTools/eng.html

Le Meleder Anna, Sauger Carine, Dubroca Laurent. 2022. Protocole de photographie des gonades de poisson / Fish gonads' photography protocol. RBE-HMMN-LRHPB. https://doi.org/10.13155/89703

ICES. 2018. Report of the Workshop for Advancing Sexual Maturity Staging in Fish (WKASMSF), 30 April - 4 May 2018, ICES Headquarters, Copenhagen, Denmark. ICES CM/EOSG: 38. 75 pp.

ICES. 2014. Report of the Workshop on sexual maturity staging of cod, whiting, haddock, Saithe and hake (WKMSGAD), 4-8 November 2013, San Sebastian, Spain. ICES CM 2013\ACOM:57. 51pp.